

## AMENDMENTS

In the specification:

Please replace the paragraph beginning at line 10 of page 20 with the following paragraph:

### EXAMPLE 1: Application to Human Squamous Carcinoma Cells

Human squamous carcinoma cells line SCC12F cells were maintained in primary keratinocyte medium (300 ml DME, 100 ml F-12 nutrient supplement, 50 ml 10x Adenine, 50 ml fetal bovine serum, 5 ml penicillin/streptomycin stock, and 0.5 ml of 10 µg/ml epidermal growth factor and hydrocortisone to final concentration of 1.4 µg/ml) and dosed with either water (diluent), 100 µM pTpT (T<sub>2</sub>, Midland Certified Reagent Company, Midland, TX) or 100 µM pdApdA (A<sub>2</sub>). Cells were harvested before dosing (day 0), and 1,3,4, and 5 days after dosage, and were counted by ~~Coulter~~ **COULTER**<sup>TM</sup> counter. After harvesting, the cells were processed for total RNA isolation and were analyzed by Northern blot. Addition of pTpT to human squamous carcinoma cells resulted in marked decreases in cell growth rate, as shown in Figure 1. Addition of a control deoxyadenine dinucleotide (pdApdA), a compound very similar to pTpT but not readily dimerized by UV irradiation and therefore rarely excised during the course of UV-induced DNA repair, has no effect.

Please replace the paragraph beginning at line 24 of page 20 with the following paragraph:

In a second experiment, SCC12F cells were cultured as described above. Two or three days after seeding, the preconfluent cultures were given fresh medium supplemented with either 100 µM T<sub>2</sub> or diluent as a control. Cells were collected daily by trypsinization and counted by ~~Coulter~~ **COULTER**<sup>TM</sup> counter. The cell yield in cultures treated with T<sub>2</sub> was reduced by 75% compared to that of paired control cultures after five days (Figure 2). This corresponds to 2.3 population doublings in this time for control cells, compared with 1 doubling for T<sub>2</sub>-treated cells.

These results further demonstrate that application of AT<sub>2</sub> DNA fragments inhibits cell proliferation, including proliferation of cancerous cells.

Please replace the paragraph beginning at line 5 of page 21 with the following paragraph:

In a third experiment, it was demonstrated that addition of T<sub>2</sub> to human squamous carcinoma cells for 24-72 hours resulted in upregulation of at least three genes: growth arrest and DNA damage (GADD 45), senescence-derived inhibitor (Sdi I), and excision repair cross-complementing (ERCC-3). Paired cultures of SCC12F cells were maintained in a Dulbecco's modified Eagle's Medium (DMEM; GIBCO/BRL, Gaithersburg, MD)-based keratinocyte growth medium supplemented with 10% fetal calf serum (Hyclone Labs, Logan, UT) and epidermal growth factor as described (Hollander, M.C. *et al.*, *J. Biol. Chem.* 268:328-336 (1992)). Pre-confluent cultures were given fresh medium supplemented with either 100 µM T<sub>2</sub>, or an equal volume of diluent. Cells were collected daily after additions and processed for total RNA isolation using the Tri-Reagent extraction method (Molecular Research Center, Cincinnati, OH) following the protocol of the manufacturer. Ten micrograms of RNA from each sample was gel electrophoresed, transferred to a nylon filter and probed as described previously (Nada, A. *et al.*, *Exp. Cell Res.* 211:90-98 (1994)). The cDNA for GADD 45 was generated by PCR using primers based on the human GADD 45 gene sequence (Mitsudomi, T. *et al.*, *Oncogene* 7:171-180 (1992)). The cDNA for ERCC 3 was purchased from the ~~American Type Culture Collection~~ **AMERICAN TYPE CULTURE COLLECTION** (ATCC, Rockville, MD). The SDI 1 cDNA was a gift of Dr. J. Smith and has been described previously (Walworth, N.C. and Bernards, R., *Science* 271:353-356 (1996)).

Please replace the paragraph beginning at line 12 of page 22 with the following paragraph:

Human cervical carcinoma cells (HeLa cells) were maintained in DME + 10% calf serum and dosed with either water (diluent) or 100  $\mu$ M T<sub>2</sub>. Cells were collected 1, 4 and 6 days after dosage and counted by ~~Coulter~~ **COULTER**<sup>TM</sup> counter.

Please replace the paragraph beginning at line 19 of page 22 with the following paragraph:

Human melanoma cell lines CRL 1424, Malma, Sk Mel 2, and Sk Mel 28 were obtained from the ~~American Type Culture Collection~~ **AMERICAN TYPE CULTURE COLLECTION** (ATCC). The cell lines were maintained in DME + 2% calf serum, and dosed with either water (diluent) with DME, or 100  $\mu$ M T<sub>2</sub> in DME. One week after dosage, cells were collected and counted by ~~Coulter~~ **COULTER**<sup>TM</sup> counter.

Please replace the paragraph beginning at line 18 of page 23 with the following paragraph:

Normal human neonatal fibroblasts were plated in Falcon P35 culture dishes at a density of  $9 \times 10^4$  cells/dish. The culture medium was DME + 10% calf serum, 2 ml per plate. One day after plating, cultures were supplemented with either 100  $\mu$ M T<sub>2</sub> in DME or 100  $\mu$ M A<sub>2</sub> in DME, or water (control). Two plates were collected and counted before the additions to give a starting, or "day 0," reading. Duplicate plates of each condition were harvested through five days after addition of the supplements and cell number determined. All cell counts were done by ~~Coulter~~ **COULTER**<sup>TM</sup> Counter. Results of two experiments, are shown in Figures 6 and 7. The results indicate that application of the DNA fragments inhibits cell proliferation.

Please replace the paragraph bridging pages 25 and 26 with the following paragraph:

Both the GADD 45 and SDI 1 genes are known to be transcriptionally regulated by the tumor suppressor protein p53. After UV and  $\gamma$ -irradiation, as well as treatment of cells with DNA-damaging chemical agents, there is a rapid stabilization and nuclear accumulation of p53 after which this protein binds to specific promoter consensus sequences and modulates the transcription of regulated genes. Recent data suggest that p53 can also be activated by the binding of small single-stranded DNAs, as well as certain peptides and antibodies, to a carboxyl terminal domain of this protein. In order to determine whether the inhibitory effect of the dinucleotide pTpT on cell proliferation is mediated through p53, the growth response of a p53 null cell line, H1299 lung carcinoma cells, was examined. The p53-null H1299 cells (Sanchez, Y. *et al.*, *Science* 271:357-360 (1996)) was maintained in DMEM with 10% calf serum. Preconfluent cultures were given fresh medium supplemented with either 100  $\mu$ M pTpT or diluent. Cells were collected on consecutive days by trypsinization, and counted by **Coulter COULTER**<sup>TM</sup> counter. As shown in Figure 9, there was no inhibition of proliferation of pTpT-treated H1299 cells compared to diluent-treated controls.

Please replace the paragraph beginning at line 16 of page 26 with the following paragraph:

In another experiment, pTpT was found to induce the expression of SDI 1 mRNA in a p53-dependent manner. Preconfluent cultures of H1299 cells were transfected with an expression vector containing the wild type human p53 cDNA under the control of the human cytomegalovirus promoter/enhancer (Dr. Bert Vogelstein, Johns Hopkins Oncology Center). Control transfections were performed using the vector from which the p53 cDNA was removed. Transfections were carried out using the Lipofectin Reagent Kit (GIBCO/BRL). One day after transfection, cells were collected for Western blot analysis using 20  $\mu$ g total protein as described (Yaar, M. *et al.*, *J Clin. Invest.* 94:1550-1562 (1994)). p53 was detected using mAb 421, anti mouse Ig linked to horseradish peroxidase (~~Amersham~~ **AMERSHAM**, Arlington Heights, IL ) and an ECL-kit (~~Amersham~~ **AMERSHAM**) following the directions of the manufacturer. At

the time of protein collection, duplicate cultures of H1299 cells transfected with the p53 expression vector (designated "p53") or control vector ("Ctrl") were given either diluent (DMEM) or 100  $\mu$ M pTpT. After 24 hours, the cells were collected, processed for RNA isolation and Northern blot analysis with an SDI 1 cDNA probe. The autoradiograph was scanned using a Macintosh IIsi computer and Macintosh One Scanner, and the brightness and contrast were adjusted to display differences in autoradiographic signals maximally. The results indicated that p53-null H1299 cells express a very low level of the SDI 1 transcript and this level is not affected by addition of pTpT. Transfection of these cells with a wild-type p53 expression vector increased the level of SDI 1 and rendered this transcript inducible by addition of pTpT. Western analysis confirmed that H1299 cells normally express no p53 and that transfected H1299 cells expressed high levels of p53. These data indicate that pTpT increases the transcriptional activity of p53.

Please replace the paragraph bridging pages 27 and 28 with the following paragraph:

Newborn keratinocytes were established as described (Stanulis-Praeger, B.M. and Gilchrest, B.A., *J. Cell. Physiol.* 139:116-124 (1989)) using a modification of the method of Rheinwald and Green (Gilchrest, B.A. *et al.*, *J. Invest. Dermatol.* 101:666-672 (1993)). First-passage keratinocytes were maintained in a non-differentiating low  $\text{Ca}^{2+}$  medium (K-Stim, Collaborative Biomedical Products, Bedford, MA). Fibroblasts were established from dermal explants as described (Rheinwald, J.G. and Green, J., *Cell* 6:331-343 (1975)) and maintained in DMEM supplemented with 10% bovine serum. Cells were treated with either 100  $\mu$ M pTpT or an equal volume of diluent (DMEM) for five days prior to transfection. Duplicate cultures of each

condition were transfected using the Lipofectin Reagent Kit (GIBCO/BRL) and 5 µg reporter DNA, pCAT-control vector (~~Promega~~ **PROMEGA**, Madison, WI). Before transfection, the vector DNA was either sham irradiated or exposed to 100 mJ/cm<sup>2</sup> UVB radiation from a 1 KW Xenon arc solar simulator (XMN 1000-21, Optical Radiation, Azuza, CA) metered at 285±5 nm using a research radiometer (model IL 1700A, International Light, Newburyport, MA), as described (Yaar, M. *et al.*, *J Invest. Dermatol.* 85:70-74 (1985)). Cells were collected 24 hours after transfection in a lysis buffer provided in the CAT Enzyme Assay System (~~Promega~~ **PROMEGA**, Madison, WI) using a protocol provided by the manufacturer. CAT enzyme activity was determined using the liquid scintillation counting protocol and components of the assay system kit. Labeled chloramphenicol [50-60 mCi (1.85-2.22 GBq) mmol] was purchased from New England Nuclear (Boston, MA). Protein concentration in the cell extracts was determined by the method of Bradford (Anal. Biochem. 72:248 (1986)). CAT activity was expressed as c.p.m./100 µg protein and is represented as percent activity of cells transfected with sham-irradiated, non-damaged, plasmid.

Please replace the paragraph beginning at line 8 of page 29 with the following paragraph:

The p53-null H1299 lung carcinoma cell line (~~American Type Culture Collection~~ **AMERICAN TYPE CULTURE COLLECTION**, ATCC, Rockville, MD) was maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL, Gaithersburg, MD) supplemented with 10% bovine serum (Hyclone Labs, Logan, UT).

Please replace the paragraph beginning at line 13 of page 29 with the following paragraph:

Preconfluent cultures of H1299 cells were transfected with an expression vector containing the wild type human p53 cDNA under the control of the human cytomegalovirus promoter/enhancer (Dr. Bert Vogelstein, Johns Hopkins Oncology Center). Control transfections were performed using the same vector lacking the p53 cDNA. Transfections were carried out as described previously. One day after transfection, cells were collected for western blot using 20 µg total protein as described. p53 was detected using the monoclonal antibody DO-1 (Ab-6) known to detect both active and inactive forms of the protein (Oncogene, Cambridge, MA), anti-mouse Ig linked to horseradish peroxidase (~~Amersham~~ **AMERSHAM**, Arlington Heights, IL) and an ECL-kit (~~Amersham~~ **AMERSHAM**) following the direction of the manufacturer.

Please replace the paragraph bridging pages 29 and 30 with the following paragraph:

Normal human keratinocytes were transfected with the human growth hormone (hGH) reporter plasmid (pPG-GH) using the Lipofectamine Reagent Kit (GIBCO/BRL) as suggested by the manufacturer and 0.5 µg pPG-GH added to each p35 culture dish. pPG-GH contains the hGH coding region under the control of the thymidine kinase (TK) promoter and p53 consensus sequence, and hGH protein production is known to be proportional to p53 activity (Kern *et al.*, 1992). Transfection was performed in the presence of 100 µM pTpT (Midland Certified Reagent Company, Midland, TX) or an equal volume of diluent. At the same time, the PSV β-galactosidase control vector (~~Promega~~ **PROMEGA**, Madison, WI) was co transfected to determine the transfection efficiency (Norton and Coffin, 1985). Four hours after transfection, medium was removed and replaced with K-Stim medium with or without 100 µM pTpT. Twenty-four hours after transfection and pTpT treatment, 400 µl of the medium was harvested from each 35 mm culture dish, and 100 µl of <sup>125</sup>I-hGH antibody solution (Nichols Institute Diagnostics, San Juan Capistrano, CA) was added to detect secreted hGH as described below. The cells were harvested in a Reporter Lysis Buffer (~~Promega~~ **PROMEGA**) using a protocol provided by the manufacturer, and 150 µl of this lysate was used for the β-galactosidase assay

using a  $\beta$ -galactosidase assay kit (**Promega PROMEGA**). Samples from each of triplicate culture dishes were evaluated for hGH and  $\beta$ -galactosidase synthesis.

Please replace the paragraph bridging pages 30 and 31 with the following paragraph:

The pCAT vector (**Promega PROMEGA**) was treated with benzo( a) pyrene-7,8-diol-9,10-epoxide (BPDE)- as described (Athas *et al. Cancer Res* 1991) to produce less damaged and more damaged plasmids, previously shown to be instructive in studies examining different repair capacities in human cells. Based on the incorporation of  $^3\text{H}$ -BPDE into the DNA, the less damaged plasmid contained 25 adducts per 5 kb plasmid and the more damaged plasmid contained 50 adducts. This non-replicating vector contains the chloramphenicol acetyltransferase gene under control of SV40 promoter and enhancer sequences. Human keratinocytes and p53-transfected H1299 cells were pre-treated with either 100  $\mu\text{M}$  pTpT or an equal volume of diluent (DMEM) alone for 48 hours, then transfected with either BP-modified pCAT-control vector (0.5  $\mu\text{g/ml}$ ) or unmodified vector (0.5  $\mu\text{g/ml}$ ) together with PSV- $\beta$ -galactosidase control vector (0.5  $\mu\text{g/ml}$ ). Cells were collected in a reporter lysis buffer (**Promega PROMEGA**) 24 hours after transfection. CAT enzyme activity was determined using the liquid scintillation counting protocol and components of the assay system kit (**Promega PROMEGA**).  $^{14}\text{C}$ -labeled chloramphenicol[50-60 mCi(1.85-2.22GBq)mmol] was purchased from New England Nuclear (Boston, MA). CAT activity was normalized with  $\beta$ -galactosidase activity.

Please replace the paragraph beginning at line 13 of page 31 with the following paragraph:

Cells were treated with 100  $\mu\text{M}$  pTpT or an equal volume of diluent alone for 48 hours. Total cellular proteins were collected in a buffer consisting of 0.25 M Tris HCl (pH 7.5), 0.375 M NaCl, 2.5% sodium deoxycholate, 1% Triton X-100, 25 mM  $\text{MgCl}_2$ , 1 mM phenylmethyl sulfonyl fluoride, and 0.1 mg/ml aprotinin. Proteins (100  $\mu\text{g}$  per sample) were separated by 7.5-15% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene,



NH). After transfer, the gel was stained with Coomassie Blue to verify even loading as visualized by the residual high molecular weight proteins. Membranes were blocked in 0.05% Tween-20/PBS with 5% milk, (Bio-Rad Laboratories, Hercules, CA). Antibody reactions were performed with the following antibodies: anti p53 (AB-6), anti PCNA (Ab-2) (Oncogene Science), and anti XPA (FL-273) (Santa Cruz Biotechnology). Sheep anti-mouse Ig linked to horseradish peroxidase (~~Amersham~~ AMERSHAM, Arlington Heights, IL) (for p53 and PCNA) and goat anti-rabbit IgG (Bio-Rad)(for XP A) were used as the secondary antibodies. Binding was detected by the ECL detection kit (~~Amersham~~ AMERSHAM).